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(57) Abstract

The invention provides methods for detecting the differential expression or presence of two analytes, and more specifically to procedures which provide for rapid and efficient analysis of gene expression in biological systems. In particular, the invention provides a method of detecting and analysing differences between nucleic acids from two sources, which method comprises: a. providing nucleic acids from two sources as labelled probes; b. forming a mixture of the labelled probes with pooled reagents wherein each reagent is a population of beads carrying a polynucleotide target, the target of one reagent being different from the target of another reagent, the beads of one reagent being distinguishable from the beads of another reagent; c. incubating the mixture under conditions to promote specific hybridisation between probes and targets; and, d. analysing beads in the mixture by flow cytometry.

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ANALYSIS OF DIFFERENTIAL GENE EXPRESSION

This invention relates to methods for detecting the differential expression or presence of two analytes, and more specifically to procedures which provide for rapid and efficient analysis of gene expression in biological systems.

Analysis of cellular gene expression is key to gaining understanding of the function of biological organisms and to elucidating the mechanisms which control key cellular events, knowledge of which is crucial to the development of drugs and strategies for treatment of disease states arising from disruption of cellular control processes.

A wide variety of methods have been developed for analysing gene expression, both at the level of mRNA expression, or by examining the amounts of specific proteins present in cells. Most methods have the same experimental basis in that they examine differential expression; that is they examine the difference in expression of one or more cellular components between two cells which have been exposed to different conditions. Such studies typically compare expression levels in cells of an untreated population (the control cells) with expression in a separate population of the same cell type (the test cells) that have been subjected to some form of stimulus, for example exposure to a hormone, drug or other chemical.

Early methods for the analysis of differential gene expression were predominantly based on analysis of (often anonymous) mRNA bands on electrophoresis gels. Such approaches have largely been displaced by more powerful, reproducible and informative methods based on the use of arrays of nucleic acids in which large numbers of specific sequences are laid down in an ordered pattern on a solid surface and form the target for hybridisation and capture of labelled mRNA or cDNA from the cells under study. Such arrays have been constructed on a variety of supports, ranging from nylon membranes to glass and silicon wafers. Whatever the support, the essential method of use is the same: firstly known sequences, complementary to cellular mRNAs, either in the form of synthetic oligonucleotides or as PCR products, are laid down on the solid support in spots at defined locations. These immobilised sequences (targets) are then exposed to sequences (probes) extracted from the cells or tissue under study, where the probes are tagged with some form of label which can be detected in subsequent analysis.

In early techniques, radioactive labels were used to probe targets on nylon membranes using techniques developed from Southern and Northern blotting methods. However these methods required the hybridisation process to be performed twice, once for the control sample and once for the test sample. They were subsequently overtaken by more elegant procedures based on the use of different coloured fluorophors to label control and test probes and using either PCR products coupled to glass (Schena M. *et al*, (1996), Proc. Natl. Acad. Sci., 93 (20), 10614-10619) or oligonucleotides synthesised directly onto glass using photo-lithographic techniques (Chee M. *et al*, (1996), Science 274 (5287), 610-61) to form what are commonly termed 'micro-arrays'.

In both methods the mRNA sequences extracted from control and test cells or tissues are either labelled directly or are first converted or amplified to yield equivalent cDNA sequences which are subsequently labelled. Once immobilised by hybridisation to complementary target sequences located on the array, the fluorescent labels attached to the probes are detected, either by scanning or by imaging, and quantified to yield data on the amounts of different mRNAs present in the test and control samples. Since sequences from test and control cells are labelled with different fluorophors, both samples can be applied and hybridised simultaneously and the resulting pattern and intensity of hybridised probes determined using detection instrumentation tuned to distinguish between the emission wavelengths of the fluorophors used.

Consequently these two-colour methods allow direct visualisation of differential expression of mRNAs between the two cell populations and are widely used in many fields of life science research to study the control and consequences of gene expression.

However, despite the relative simplicity and elegance of these methods, in operation they suffer from a number of technical difficulties which limit the ease of application and the speed of the techniques to the analysis of gene expression. The construction of target arrays is a time consuming and often expensive process requiring precision equipment: either for construction and alignment of the masks required for light directed oligonucleotide synthesis, or for precise application of nanolitre droplets of liquid for DNA arrays. Arrays based on DNA spots also suffer from artefacts arising from unequal application or drying of the very small volumes used, and often require

replicate spots to yield accurate data. With either type of array, if the user wishes to modify his investigations to include additional sequences a whole array must be constructed to accommodate the new targets. It would not be atypical to have to make an entirely new array simply to add a single new sequence to a pre-existing library of several thousand sequences.

The hybridisation process is also subject to a number of problems arising from the geometry of the system and the temperature required for the hybridisation process. To achieve sufficient sensitivity to detect low levels of mRNAs it is necessary to use a high concentration of labelled probe to achieve maximal hybridisation to target sequences; this requirement and the limited amount of probe material available results in hybridisation reactions being performed in very small volumes. Difficulties therefore arise in ensuring adequate coverage of arrays with microlitre quantities of solution, and the resulting thin films of liquid do not promote good access of the mobile probe sequences to the fixed target sequences; furthermore problems with evaporation are also common at the temperatures of 40°C to 65°C commonly used for hybridisation.

Finally, the detection and quantification of fluorophor-labelled probes on micro-arrays requires dedicated sophisticated equipment to detect the very low levels of fluorescence present. To achieve the required sensitivity; detection is most commonly achieved using a scanning laser spot to excite fluorophor molecules; this can be a very slow process requiring up to several hours to complete measurements from a single array.

This invention provides an alternative to micro-array systems for analysis of gene expression. Means are provided for performing analyses using a particle based technique so as to replace ordered 2D arrays with randomly oriented 3D arrays which can be quickly and easily modified to include new target sequences. The technique provides favourable geometry and kinetics for promoting efficient hybridisation, that can be performed in a standard reaction tube, and that allows measurement of hybridised probe to several thousand target sequences to be accomplished in a few seconds.

The invention provides a method of detecting and analysing differences between nucleic acids from two sources, which method comprises:

a. providing nucleic acids from two sources as labelled probes;

b. forming a mixture of the labelled probes with pooled reagents wherein

each reagent is a population of beads carrying a polynucleotide target, the target of one reagent being different from the target of another reagent, the beads of one reagent being distinguishable from the beads of another reagent;

c. incubating the mixture under conditions to promote specific hybridisation between probes and targets; and,

d. analysing beads in the mixture by flow cytometry.

A polynucleotide target is partly or wholly single-stranded and is capable of specific hybridisation. Oligonucleotides of at least 8 residues are preferred. Preferred are cDNA sequences derived, e.g. by RT-PCR amplification, from cellular mRNA.

The pooled reagents may comprise one bead, or preferably a plurality of beads, of each reagent.

Features of the invention include:

a) gene expression assays are performed on carrier beads;

b) individually identifiable beads or populations of beads each carrying a different target sequence are prepared;

c) selected beads, or populations of beads, are pooled together in suspension to provide a randomly oriented 3D array of particles carrying all sequences of interest for an individual investigation;

d) mRNAs or cDNAs prepared from control and test cells or tissues are labelled with fluorescent tags to identify their source;

e) labelled probe species are mixed with the pooled suspension of target carrying beads under conditions which promote specific hybridisation between probes and targets;

f) the bead mixture is analysed by flow cytometry to simultaneously determine the identity of each bead analysed (and hence the identity of the target sequence carried by the bead) and to quantify the amounts of both control and test probes bound to each bead; and

g) data is analysed to yield information on the relative and absolute abundance of each mRNA in the control and test samples.

The beads of one reagent can be distinguished from the beads of another reagent by a number of different means. Suitable distinguishing means include differences in size, colour or fluorescence or the nature or concentration of markers

attached to the beads. Beads of one reagent can be distinguished from beads of other reagents using one or more of such means.

For clarity, the invention is now described with reference to the following figures:

Figure 1: Schematic illustration of 2D ordered array and 3D random array.

Figure 2: Flowchart illustrating the principle of the bead based flow cytometry gene expression process.

Figure 3: Schematic representation of the bead based flow cytometry gene expression process.

Figure 4: Schematic representation of method for analysis of differential expression of TNF and GAPDH genes in LPS stimulated THP-1 cells.

With reference to Figure 1, in a two dimensional ordered array the locations of each target spot immobilised on a planar surface are defined by x,y co-ordinates and hence target sequences are identified by the same co-ordinates. In contrast, in a 3D array formed from particles dispersed in a space defined by dimensions x,y,z, if each particle is individually identifiable by some inherent characteristic, it is not necessary to use x,y,z locations to specify the identity of each bead and the particles can be randomly distributed throughout the volume as in a suspension of beads in liquid. It follows that if each bead is individually identifiable, then any target sequence previously coupled to that bead is also identifiable. Therefore if a number of differing beads, or discrete populations of beads, are individually prepared where each bead carries a different target sequence and then selectively pooled, the pooled beads can form a 3D array which can be used for gene expression analysis.

Beads suitable for use in the method of the invention are those which can be readily identified during analysis by flow cytometry: such beads have been previously developed and used for diagnostic assays to measure a wide range of analytes in blood and other biological fluids by immunoassay. A desire to have a higher throughput in these applications has led to the development of multiplex methods which allow more than one analyte to be measured simultaneously by means of flow cytometry analysis. Multiplexing is achieved by carrying out solid phase linked assays

using plastic or latex beads as assay substrates. By using a number of discrete bead types which are individually distinguishable from each other, where each bead type carries reagents for one assay, standard flow cytometer instrumentation may be used both to identify the bead type and to measure the assay signal associated with each bead. Discrimination between bead populations can be achieved by size (Frengen J. *et al* (1995), Journal of Immunological Methods, Volume 178, p141), by colour or fluorescence (Fulwyier M.J. UK Patent 1,561,042) or by electronic means (Mandecki W. US Patent 5,641,634).

The general principle of the process of the invention is now described with reference to Figure 2. Selected target cDNA sequences are prepared by standard PCR methods incorporating a means to allow coupling of target sequences to beads. One suitable method would utilise a 5'-biotin on one of the PCR primers, yielding a 5'-biotinylated DNA suitable for coupling to streptavidin-coated beads. Those skilled in the art will recognise that alternative chemical coupling strategies are available. Such alternative strategies may include, for example, synthesising oligonucleotides having a chemical group such as an amino group at the 5' end thus rendering them suitable for crosslinking to beads which have been modified to have, for example, carboxyl groups on their surface. It will also be appreciated that oligonucleotides synthesised with a terminal biotin or other coupling group could readily be used in place of PCR generated DNA sequences.

Once the required number of target cDNAs (cDNA 1 to cDNA n) have been prepared, each target sequence is separately coupled to a corresponding discrete population of beads (Bead 1 to Bead n respectively). Aliquots are then removed from each population and pooled to form a mixed suspension of beads constituting a randomly orientated 3D array of target sequences. The 3D array is then hybridised with fluorescently labelled probes (RNA or cDNA) prepared from the control and target cells or tissues which have been labelled with two different fluorophors (Fluor A and Fluor B respectively). Following hybridisation the mixed population of beads is analysed by flow cytometry; as each bead is analysed information from the flow cytometer detectors is used to identify the bead and to measure the amounts of Fluor A (control mRNA) and Fluor B (sample mRNA) bound to the complementary target sequence carried by the bead. These measurements are then used to determine the relative expression of each

mRNA in the samples.

In a further illustration of the method of the invention reference is made to one embodiment as shown in Figure 3. Control (1) and test (2) probes are prepared and labelled using standard methods and aliquots mixed in a tube (3) containing a mixture of beads (4) carrying the desired target sequences and the tube sealed. Hybridisation of probe and target sequences are promoted by incubating the mixture under conditions of heat, pH and salt concentration which are known to allow the formation of specific nucleic acid hybrids. Following hybridisation, the bead mixture is analysed by flow cytometry using multiple channel fluorescence detection. In the embodiment illustrated, two fluorescence channels are used to identify beads and two further channels are used to measure control and test probe fluorescence. For each bead passing through the flow cytometer this data produces a set of data values that can be represented as 3D plots for control probes (7) and test probes (6). Bead identity is determined by measuring the amounts of two different fluorophors (bead Fluor 1 and bead Fluor 2) incorporated within the bead during manufacture. Plotting the intensities of the two fluorophors on x,y axes (8 & 9) separates the different bead populations used.

The number of possible target sequences that can be measured in a single assay will necessarily be limited by the number of bead populations which it is possible to discriminate in a mixture. With current flow cytometry instrumentation this does not pose a limitation on the utility of the procedure. Typical modern flow cytometry instruments are capable of simultaneously measuring fluorescence at four wavelengths together with other parameters, for example light scattering which is a measure of the size of particles under analysis. In addition, the dynamic range of fluorescence detection is high and fluorescence may be accurately measured over several orders of magnitude. Given this sophistication in measurement it is relatively straightforward to devise schemes which yield a large number of individually distinguishable bead populations to serve as carriers. For example if beads are prepared which contain 2 separate fluorophors, with each fluorophor present in one of 10 levels, then $10^2 = 100$ bead types are created. By increasing the number of fluorophors, or the levels of each fluor or by introducing other variables, such as bead size, larger numbers of discrete bead types can be produced.

Plotting the intensity of probe fluorophors on the z axis of two different

plots (10 & 11) shows the amounts of the control (10) and test (11) probes bound to each bead population. This allows the production of a table of the amounts bound to each target sequence in the analysis (14). In the schematic example shown, one mRNA species (12) is expressed at a lower level in the test sample than in the control (13). Other differences in expression can be readily identified as differences in the heights of equivalent peaks in the two plots.

In micro-array technique applications DNA or oligonucleotide target sequences are typically applied to a solid surface as discrete areas of dimensions in the range 10-100 μm, with dimensions of 50-100 μm being typical of DNA spots applied as liquid droplets, and smaller areas being used in techniques utilising photo-lithographic oligonucleotide synthesis. To ensure accuracy in measurement of differential expression it is important that the amount of DNA or oligonucleotide present on the solid phase is in excess of complementary sequences in the probe solution such that target sequences do not become limiting leading to distortion of hybridisation results. Consequently it is crucial that in any procedure using flow cytometry for analysis of gene expression using target sequences carried on beads, that the capacity of the system retains the same degree of target:probe excess as used in conventional techniques. Beads used for flow cytometry typically have diameters in the range from 1-10 μm and therefore individually do not have sufficient surface area to substitute for a typical micro-array. However by using several beads to carry each target sequence it is possible to achieve equivalence in target presentation as shown in the following example:

For a 2D array with 1 00 μm ∅ spots:

spot area $= \pi r^2$

$= 3.14 \times (50)^2$

$= 7850\ \mu m^2$

For 10 μm ∅ beads in beam (50% surface illuminated):

lit area $= 2\pi r^2$

$= 2 \times 3.14 \times (5)^2$

$= 157\ \mu m^2$

Bead:Spot equivalence = 7850 / 157
= 50 beads

Bead volume (assuming cubic packing) = 10^3 μm^3
Volume of 50 beads = 50,000 μm^3
Assuming 10% v/v suspension = 500,000 μm^3
= $5 \times 10^5 / 1 \times 10^9$ μl
= 1×10^{-3} μl
= 1 nl

Consequently for a complete assay of 1000 targets using 50 beads/target the total volume required for the assay is 1 µl. If desired, larger volumes may be used for convenience in hybridisation or analysis; for example using beads at a concentration of 1 % v/v would give a 10 µl total volume.

If desirable, the method of the invention would allow larger numbers of beads to be used with a consequent increase in capacity for binding a greater mass of target sequence spread over the total bead population. This would allow the user if desired to increase the amount of probe bound to the bead populations to increase the sensitivity of the process for detecting rare species. Alternatively, it enables an increase in the number of samples which may be analysed simultaneously, for example, to measure expression of a panel of genes simultaneously in a control and more than one test sample, where as described previously each control or test sample is labelled with a different fluorophor. Such increases in assay complexity are not achievable with conventional arrays on solid surfaces without reducing sensitivity due to the finite capacity of array spots for binding complementary sequences.

It can be appreciated by the skilled worker that the method of the present invention provides a number of significant advantages over previously described procedures for gene expression analysis which are based on 2D arrays:

a) the basic components for the bead based assay are readily prepared by coupling solutions of cDNA or oligonucleotides to commercially available beads using standard coupling methods,

b) no specialised equipment is required for preparation or analysis, in contrast to the dedicated array production and scanning equipment required for micro-arrays,

c) the design of investigations can be easily modified with target sequences being added or deleted at will without the requirement to scrap existing materials,

d) hybridisation is performed in suspension in standard reaction vessels, thereby avoiding problems with evaporation associated with thin films of liquid covering micro-arrays and promoting hybridisation through efficient mixing of probe and target sequences, and

e) analysis speed is significantly improved: flow cytometers typically analyse beads at rates of 1,000-10,000 beads/second allowing processing of a 100 sequence gene expression analysis in a few seconds.

In a further illustration of the method of the invention reference is made to one embodiment as shown in Figure 4.

Figure 4 shows a scheme of a method for the analysis of differential Tumour Necrosis Factor (TNF) expression in THP-1 cells either treated in the presence (test) or absence (control) of bacterial lipopolysaccharide (LPS) (Su S. etal BioTechniques 1997, 22:1107-1113). In this method, variations in abundance of the TNF gene transcripts in control and test samples are simultaneously compared to levels of transcripts from the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene in the same samples.

Briefly, human monocyte THP-1 cells at 5 x 10^5 cells/ml are treated in the presence (test sample) and absence (control sample) of 10µg/ml LPS for 90 minutes. Following treatment, both samples are processed separately to isolate the RNA populations present in the control and test samples according to standard procedures set out in Molecular Cloning, A Laboratory Manual, Cold Spring Harbour Laboratory Press 1989, pp 7.3-7.87. Other suitable methods for RNA isolation will be recognised by someone skilled in the art and include the use of commercially available reagents or kits (e.g. RNeasy, Qiagen).

Following RNA isolation, mRNA molecules are converted to cDNA by means of the enzyme, reverse transcriptase, using the standard method set out in Molecular Cloning, A Laboratory Manual, Cold Spring Harbour Laboratory Press 1989,

pp 8.11-8.13.

The cDNA molecules obtained from each sample are then used in separate multiplex PCR reactions using standard conditions (Molecular Cloning, A Laboratory Manual, Cold Spring Harbour Laboratory Press 1989 pp 14.5-14.20) and primer pairs which amplify GAPDH and TNF (Su S. etal BioTechniques 1997, 22:1107-1113). One primer of each primer pair is modified at the 5' end with a biotin molecule to aid in strand separation in subsequent steps in the process. This is readily achieved using standard techniques during oligonucleotide synthesis with modified phosphoramidites, for example : 5'-Biotin Phosphoramidite (Glen Research). Fluorescently labelled nucleotides are incorporated into the PCR reactions in order to label the amplified PCR products such that their origin from the test or control RNA populations may be established. Thus, the PCR reaction mix containing cDNAs prepared from the control cells includes Cy3™-dCTP and the reaction mix containing cDNAs prepared from test cells includes Cy5™-dCTP (Cy3™-dCTP and Cy5™-dCTP are obtained from Amersham Pharmacia Biotech). These reactions yield amplified levels of fluorescently labelled cDNA populations wherein the relative abundance of individual cDNA species corresponds to the abundance of their respective parent mRNA species in the RNA populations isolated from the cell or tissue samples and where the origin of any given molecule from the test or control sample populations can be ascribed from the fluorescent label carried by the molecule.

The PCR products from the control (Cy3-labelled) and test (Cy-5 labelled) reactions are now mixed together prior to further processing.

Double stranded cDNA products of the PCR reactions are converted to single stranded cDNA molecules prior to further analysis by binding the PCR products via the biotin at the 5' end of each PCR product to streptavidin-coated magnetic beads (MagneSphere, Promega). Once bound, the double stranded PCR products are denatured by addition of 0.2 volume of 2M NaOH, incubating for 10 minutes at room temperature in order to release the non-biotinylated strands from the beads. The beads are separated from the solution by attraction to a magnet and the clarified solution, which contains the single stranded labelled PCR products, is removed and made neutral by addition of 1 volume of 0.4M HCl. Other methods for separating double-stranded DNA molecules are known to those skilled in the art.

Populations of beads or particles suitable for performing analysis of

differential gene expression are prepared using beads containing fluorescent dyes (SPHERO™, Spherotech Inc.). Yellow fluorescent streptavidin-coated beads are coated with the 5'-biotin primer for GAPDH and blue fluorescent streptavidin-coated beads are coated with the 5'-biotin primer for TNF (using the same 5'-biotin primers as were used earlier). Thus, these populations of beads have two distinguishing characteristics i.e. each bead population is distinguishable from all other bead populations by its fluorescence characteristics and each bead population is capable of recognising a single cDNA species (either TNF or GAPDH).

The separately prepared beads are mixed together, further mixed with the Cy 3 and Cy 5-labelled single stranded cDNA products prepared previously and incubated in a hybridisation buffer (0.1M Tris.HCl pH7.4, 750mM NaCl) at 45° for 2 hours prior to analysis of the bead populations by flow cytometry. cDNA products from the mixed population will bind specifically to their complementary capture sequences carried on beads such that each bead will ultimately be decorated with a mixture of labelled cDNAs of a single species arising from the test and control populations, and where the relative abundance of the labels reflects the relative abundance of single species in the original test and control populations.

The relative abundance of GAPDH transcripts in the population of molecules derived from control and test samples is compared by detecting the fluorescence of yellow beads at 460nm/ 480nm (excitation/emission); and, within the population of yellow beads, bound Cy3 labelled transcripts are detected at 550nm/570nm (excitation/emission) and bound Cy5 labelled transcripts are detected at 650nm/670nm (excitation/emission). The relative abundance of TNF transcripts in the molecules derived from test and control samples is determined by detecting the fluorescence of blue beads at 650nm/710nm (excitation/emission); and further detecting bound Cy3 labelled transcripts at 550nm/570nm (excitation/emission) and bound Cy5 labelled transcripts at 650nm/670nm (excitation/emission). Thus bead fluorescence is used to assign the identity of the gene associated with each bead and label fluorescence is used to determine the relative abundance of cDNAs arising from the test and control samples which are attached to the bead, the latter yielding information on the differential expression of that gene under the conditions used in treatment of the original cell or tissue sample.